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Published in:
Journal of Veterinary Diagnostic Investigation

Link to article, DOI:
[10.1177/1040638712452111](https://doi.org/10.1177/1040638712452111)

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Pedersen, K. S., Pedersen, K. H., Hjulsager, C. K., Larsen, L. E., Ståhl, M., Stege, H., Angen, Ø., & Nielsen, J. P. (2012). Within-day repeatability for absolute quantification of *Lawsonia intracellularis* bacteria in feces from growing pigs. *Journal of Veterinary Diagnostic Investigation*, 24(5), 968-970.
<https://doi.org/10.1177/1040638712452111>

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Journal of Veterinary Diagnostic Investigation

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J VET Diagn Invest 2012 24: 968 originally published online 11 July 2012

DOI: 10.1177/1040638712452111

The online version of this article can be found at:

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Journal of Veterinary Diagnostic Investigation
24(5) 968–970
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sagepub.com/journalsPermissions.nav
DOI: 10.1177/1040638712452111
http://jvdi.sagepub.com

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Abstract. Absolute quantification of *Lawsonia intracellularis* by real-time polymerase chain reaction (PCR) is now possible on a routine basis. Poor repeatability of quantification can result in disease status misclassification of individual pigs when a single fecal sample is obtained. The objective of the current study was to investigate overall variation within a day for fecal numbers of *L. intracellularis* bacteria determined by real-time PCR in growing pigs. From each of 30 pigs with an infection of *L. intracellularis*, 5 fecal samples were collected within 1 day. A total of 150 fecal samples were obtained and subjected to quantitative PCR (qPCR) testing. Mean fecal dry matter content was 14.3% (standard deviation = 4.5%). Two pigs (6.7%) alternated between being *L. intracellularis* qPCR positive and negative. For 28 pigs, the excreting levels of *L. intracellularis* were within the dynamic range of the qPCR assay at all sampling points. For these 28 pigs, the mean excretion level of *L. intracellularis* was $6.1 \log_{10}$ bacteria/g feces (standard deviation = $1.2 \log_{10}$ bacteria/g feces). The maximum observed difference between 2 fecal samples from the same pig was $1.1 \log_{10}$ bacteria/g feces. The average standard deviation for individual pigs was $0.27 \log_{10}$ bacteria/g feces. The average coefficient of variation within pigs was 0.04, ranging from 0.006 to 0.08. The results imply that absolute quantification of *L. intracellularis* by qPCR has acceptable repeatability within 1 day. However, a population-specific proportion of pigs alternating between positive and negative test results must be expected.

Key words: Fecal samples; *Lawsonia intracellularis*; pigs; quantitative real-time polymerase chain reaction; within-day variation.

Lawsonia intracellularis is an obligate intracellular bacterium associated with intestinal disease in growing pigs.² It is considered among the most economically important infections in the swine industry worldwide.¹ Routine culture methods are not possible for *L. intracellularis*; therefore, testing of fecal samples by qualitative polymerase chain reaction (qPCR) has been applied for diagnosis of *L. intracellularis*-associated disease in diagnostic laboratories around the world.⁵ However, interpretation of these traditional qualitative fecal PCR tests is difficult in terms of importance for the individual pig.⁵ Real-time qPCR tests have been applied for absolute quantification of bacteria in clinical samples involving other agents, and a correlation between excretion level and disease severity has been demonstrated.⁴ Furthermore, clinically important levels for bacterial load have been established in other diseases.⁸ In 2009, qPCR tests were developed for absolute quantification of *L. intracellularis* in fecal samples.³ Absolute quantification of *L. intracellularis* by qPCR could potentially enhance interpretation of fecal PCR test results if numbers of *L. intracellularis* bacteria in feces and disease severity are correlated. Absolute quantification of *L. intracellularis* in routine diagnostics requires a high repeatability and reproducibility for quantification of clinical samples. A poor repeatability/reproducibility could potentially

result in misclassification in relation to disease status when a single fecal sample is obtained from an individual. Repeatability/reproducibility for absolute quantification by qPCR can be influenced by features inherent to the qPCR assay (intra- and interassay variation) and biological variation (i.e., homogeneity of the fecal samples [intrasample variation] and variation in excretion level between fecal samples obtained from the same individual within the same day [within-day variation]). The objective of the current study was to investigate overall variation within a day for fecal absolute quantification of *L. intracellularis* by real-time PCR in growing pigs.

A cross-sectional study with follow-up was performed. No published reports of within-day variation of *L. intracellularis* in fecal samples were available for sample size calculations.

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A working sample size for the study was based on the practical aspects of collecting the fecal samples. One person should collect all fecal samples. It was considered possible to obtain fecal samples from approximately 30 pigs in 1–1.5 hr. A Danish farrow-to-finish herd with a history of *L. intracellularis* infection was selected by purpose sampling. The herd was selected based on a previous history of *L. intracellularis*. A total of 30 pigs were selected at convenience from a batch of 350 pigs that were approximately 8 weeks old having an outbreak of diarrhea. Based on previous laboratory herd examinations, the pigs were expected to be naturally infected by *L. intracellularis*. The selected pigs had not received antibiotic treatment within the last 4 weeks, and treatment of the current diarrhea outbreak was not initiated until after the fecal samples were collected. Selected pigs were identified by 2 identical ear tags. Fecal samples (10–50 g/sample) were collected 5 times during 1 day (at 9 AM, 11 AM, 1 PM, 3 PM, and 5 PM) from the rectum of each pig using a new plastic glove for each pig. At each collection time, the pigs were handled in the same chronological order. All fecal samples were kept at 4°C and further processed the following day.

Fecal samples were mixed with a spoon and diluted to 10% in phosphate buffered saline (PBS). Total DNA was extracted using a commercial kit.^a One negative extraction sample of other bacterial cells and 1 positive extraction sample of *L. intracellularis* were included in each experiment. The amount of *L. intracellularis* in feces was determined with a qPCR termed the Laws-qPCR assay.⁷ For absolute quantification of *L. intracellularis*, a standard curve was made from 10% pig feces, negative by qPCR and spiked with a 10-fold dilution of the reference strain *L. intracellularis* (strain 15540) as earlier described.⁷ Each qPCR experiment included the same reference concentrations of pure DNA in triplicate and facilitated adjustment of the standard curves to each new qPCR run.⁷ The dynamic range of the Laws-qPCR was 4×10^3 to 4×10^8 bacteria/g feces, and the limit of detection was 10^2 bacteria/g feces. All fecal samples were subjected to fecal dry matter (DM%) determination in a microwave oven as previously described.⁶

Prior to data analysis, qPCR results were \log_{10} transformed to obtain a normal distribution. Initial descriptive analysis was performed using different summary statistics and plots. Overall variation for the individual pigs was evaluated by calculation of the standard deviation (SD) and coefficient of variation (CV). Trends for SD in relation to increasing mean excretion levels in the individual pigs were investigated by linear regression. Commercial statistical software^b was used for all analyses.

A total of 150 fecal samples for qPCR testing were obtained. Mean fecal dry matter content was 14.3% (SD = 4.5%). All pigs had a minimum of 1 qPCR-positive fecal sample. Two of the pigs were *L. intracellularis* qPCR negative in 1 and 3 of the fecal samples, respectively. One of these pigs alternated from below to above the limit of

detection, while the other pig was below the limit of detection in 1 sample, within the dynamic range in 1 sample, and above the limit of detection but below the dynamic range in 3 samples.

The results from these 2 pigs could not be included in the statistical analyses of overall quantitative variation because of the missing values of *L. intracellularis* excretion level. A total of 28 pigs were excreting *L. intracellularis* at a level within the dynamic range of the Laws-qPCR assay at all time points. A total of 140 fecal samples obtained from these 28 pigs were included in the statistical analysis. The mean excretion level of *L. intracellularis* was $6.1 \log_{10}$ bacteria/g feces (SD = $1.2 \log_{10}$ bacteria/g feces). The maximum observed difference between 2 fecal samples from the same pig was $1.1 \log_{10}$ bacteria/g feces. The average SD for individual pigs was $0.27 \log_{10}$ bacteria/g feces. A linear association between SD and mean excretion level was not demonstrated ($P = 0.37$). The average CV within pigs was 0.04, ranging from 0.006 to 0.08.

For fecal qPCR quantification of *L. intracellularis*, 95% of all samples from an average pig can be expected to be within approximately $1 \log_{10}$ bacteria/g feces. Furthermore, the probability of observing a value more than $0.5 \log_{10}$ bacteria from the true excretion level is below 0.025. The results imply that absolute quantification of *L. intracellularis* by real-time PCR has a good repeatability within 1 day. Therefore, qPCR has the potential to be applied as a quantitative test for diagnosis of *L. intracellularis*-associated disease in individual pigs.

However, 2 pigs alternated from below to above the limit of detection in the current study. This illustrates that samples obtained at different time points can result in different classification of individuals in relation to infection and/or disease status. The 2 pigs were probably having a true excretion level close to the limit of detection. Therefore, the overall variation between samples within the same day would result in test results being above or below the limit of detection from one examination to the next. However, this problem is not specifically associated to absolute quantification by PCR tests. This problem of classification bias is relevant for any PCR test or culture method from fecal samples. The proportion of pigs alternating between positive and negative test results will depend on the fecal level of bacteria in the specific population of pigs.

Absolute quantification of *L. intracellularis* by real-time PCR has an acceptable repeatability within 1 day. However, a population-specific proportion of pigs alternating between positive and negative test results must be expected, resulting in false-negative disease classification.

Sources and manufacturers

- QIAamp DNA Stool Mini Kit, Qiagen GmbH, Hilden, Germany.
- Stata/IC version 11, StataCorp LP, College Station, TX.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was financially supported by Boehringer Ingelheim, Denmark.

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